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Molecular Typing of Ampicillin-Resistant, Non-β-Lactamase-Producing *Enterococcus faecium* Isolates from Diverse Geographic Areas

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Molecular typing methods were compared by using 66 ampicillin-resistant, non-β-lactamase-producing *Enterococcus faecium* clinical isolates from diverse geographic areas. Whole-plasmid analysis, restriction enzyme analysis of plasmid DNA with *Eco*RI and *Hin*dIII, and contour-clamped homogeneous electric field electrophoresis with digestion by *Sma*I and *Apa*I were performed on all isolates. Whole-plasmid analysis identified 47 different groups. Restriction enzyme analysis of plasmid DNA identified 50 groups when *Eco*RI was used and 51 groups when *Hin*dIII was used. Results with *Eco*RI and *Hin*dIII differed in 9 of 66 isolates. Grouping results with whole-plasmid analysis differed from results of restriction enzyme analysis of plasmid DNA (combining *Eco*RI and *Hin*dIII) in 20 of 66 isolates. Contour-clamped homogeneous electric field electrophoresis identified 46 groups when *Sma*I was used and 44 groups when *Apa*I was used. Results with *Sma*I and *Apa*I differed in 3 of 66 isolates. Grouping results with contour-clamped homogeneous electric field electrophoresis (combining *Sma*I and *Apa*I) differed from results of restriction enzyme analysis of plasmid DNA (combining *Eco*RI and *Hin*dIII) in 17 of 66 isolates. The combined use of whole-plasmid analysis, restriction enzyme analysis of plasmid DNA with two enzymes, and contour-clamped homogeneous electric field electrophoresis with two restriction enzymes should be considered when *E. faecium* is typed for epidemiologic investigation.

Enterococci have emerged in recent years as pathogens in a growing number of serious nosocomial infections including bacteremia and intraabdominal and urinary tract infections (9, 12, 13, 18, 19). Especially worrisome are the increased numbers of enterococcal isolates that are resistant to the aminoglycosides, penicillins, or glycopeptide agents (1, 2, 6, 7, 10, 13, 17, 18, 22, 24, 27). The spread of these antibiotic-resistant enterococcal strains has occurred not only within individual hospitals but also between hospitals of varied geographic locations across the United States (15, 21, 25, 26). The proportion of enterococcal isolates that exhibit clinically important antimicrobial resistance has become quite high at some chronic-care facilities as well as at acute-care hospitals (1–3, 6, 10, 17, 21, 25, 27). Epidemiologic studies of enterococcal infections have been hindered because the most useful DNA typing method or combination of methods to reliably and conveniently differentiate enterococcal strains has not been adequately identified and substantiated in practice. Antibiograms are now completely inadequate. Plasmid analysis has been used in many institutions for typing enterococci, but there is a growing awareness that this method used alone is insufficient (14). In this study, contour-clamped homogeneous electric field electrophoresis (commonly referred to as CHEF electrophoresis) of genomic DNA is compared with the more-traditional methods of agarose gel electrophoresis of whole-plasmid DNA and restriction enzyme-digested plasmid DNA as applied to 66 ampicillin-resistant, non-β-lactamase-producing *Enterococcus faecium* isolates from diverse geographic areas. The purpose of the comparison is to evaluate the molecular relatedness of the isolates by these techniques to determine which method or methods might be most useful for reliable strain differentiation and to obtain evidence about geographic dispersion of strains.

MATERIALS AND METHODS

Strains. Sixty-six clinical isolates of ampicillin-resistant, non-β-lactamase-producing *E. faecium* from separate patients in California (15 isolates), Virginia (13 isolates), Illinois (9 isolates), Michigan (9 isolates), Rhode Island (6 isolates), North Carolina (5 isolates), Toronto, Canada (5 isolates), Georgia (2 isolates), South Carolina (1 isolate), and Vermont (1 isolate) were studied. Isolates from California (susceptibility testing results) and Rhode Island (clinical epidemiology and DNA typing results) have been described previously (1, 17). Conventional biochemical reactions were

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used to identify the organisms (5). Susceptibility to ampicillin (Sigma Chemical Co., St. Louis, Mo.) was determined by a standard microdilution method (16). An isolate was considered ampicillin resistant if the MIC of ampicillin was ≥16 μg/ml. Isolates were screened for production of β-lactamase by using nitrocefin disks (BBL Microbiology Systems, Cockeysville, Md.). Isolates were from epidemiologically related and unrelated patients. No two isolates came from the same patient. The hospitals were composed of acute-care community, veterans' affairs, and university medical centers used for primary through tertiary care.

Plasmid and genomic-DNA preparation. Plasmid DNA was prepared by modifications of a previously described method (4). All reagents were from Sigma unless otherwise noted. Brain heart infusion broth (100 ml; Difco Laboratories, Detroit, Mich.) was inoculated with 1 ml of an 18-h culture of the isolate and incubated for 3 h in a 37°C shaking water bath. Three grams of glycine was added, the culture was reincubated for 1 h, and cells were collected by centrifugation. The cells were suspended in 5 ml of 25% sucrose in TES (0.5 M Tris, 45 mm EDTA, 0.05 M NaCl); 1 ml of freshly prepared 5-mg/ml lysozyme in TES and 50 U of mutanolysin were added; and the solution was incubated for 30 min in a 37°C shaking water bath. Two milliliters of 0.25 M EDTA (pH 8.0) was added, the solution was incubated for 15 min at 37°C, 1 ml of 5-mg/ml preincubated pronase was added, and the mixture was incubated for 30 min at 37°C. Seven milliliters of 2% sarcosine in TES was added, and the DNA was sheared with a pipette. The solution was brought up to 30 ml with TES, and 30 g of cesium chloride (BRL Life Technologies, Inc., Gaithersburg, Md.) was added. The solution was then transferred to a centrifuge tube containing 0.5 ml of 10-mg/ml ethidium bromide and ultracentrifuged (100,000 × *g*, 20 h) to separate plasmid from chromosomal DNAs. The plasmid band was collected under UV light, decolorized with cesium chloride-saturated isopropanol, and dialyzed in 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) for 18 h. The DNA was precipitated by adding 1/10 its volume of 3.0 M sodium acetate and 2 volumes of ethanol and kept at −20°C for 18 h. For whole-plasmid analysis, 1 μg of DNA was loaded onto a 0.7% agarose vertical gel in TAE (0.4 M Tris, 0.2 M Na citrate, 9 mM EDTA, pH 8.2), run for 2 h at 80 V, and stained in ethidium bromide. For restriction enzyme analysis (REA), 1 μg of plasmid DNA was digested with 10 U of *Eco*RI (BRL) or 10 U of *Hin*dIII (BRL) according to the manufacturer's directions and run on a vertical gel as described above.

Genomic DNA was prepared by modifying a previously described procedure (14, 23). Cells were grown in 5 ml of brain heart infusion broth for 18 h and collected by centrifugation. The cell pellet was suspended in 2 ml of PIV buffer (1 M NaCl, 10 mM Tris, pH 7.6) and mixed with 2 ml of 1.6% low-melting-point agarose (BRL) at 55°C. The mixture was transferred into sample plug molds (Bio-Rad Laboratories, Richmond, Calif.) and refrigerated for 1 h. Sample plugs were incubated at 37°C for 18 h in 5 ml of lysis buffer (6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% deoxycholate, 0.5% sarcosine, 20 μg of RNase per ml, 1 μg of lysozyme per ml, pH 7.6), incubated at 55°C for 18 h in 5 ml of ESP solution (0.5 M EDTA [pH 9 to 9.5], 1.0% sarcosine, 50 μg of proteinase K per ml), incubated with 5 ml of TE (10 mM Tris, 0.1 mM EDTA, pH 7.5) at 37°C three times (30 min each time), and stored in TE at 4°C. One-quarter of a sample plug was placed in a microfuge tube containing 200 μl of sterilized deionized water, 25 μl of React 4 buffer (BRL), and 30 U of *Sma*I (BRL) or 30 U of *Apa*I (BRL) and incubated at room temperature for 6 h. The plug was then incubated in 1 ml of TE at 37°C for 1 h. Sample plugs were loaded in a 0.8% agarose gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA), electrophoresed on a CHEF DR II apparatus (Bio-Rad) (initial switch time, 5 s; final switch time, 35 s; start ratio, 1; voltage, 200; run time, 23 h; temperature, 4°C), and stained with ethidium bromide. Strains were differentiated by visual inspection. Isolates were placed in different groups if they differed by even one band. For any two isolates to be considered members of a single group with REA of plasmid DNA, their patterns had to be identical by both *Eco*RI and *Hin*dIII analysis; with CHEF electrophoresis of genomic DNA, they had to be identical by both *Sma*I and *Apa*I analyses.

RESULTS

With whole-plasmid analysis, plasmid profiles of the 66 *E. faecium* isolates allowed us to place the isolates into 47 groups. Twelve groups contained more than one isolate (ranging from two to five isolates). REA of plasmid DNA combining results with both *Eco*RI and *Hin*dIII identified 56 groups. Six of the 56 groups contained more than one isolate (ranging from two to five isolates). The use of *Eco*RI alone identified 51 groups, and the use of *Hin*dIII alone identified 51 groups; grouping results with *Eco*RI and *Hin*dIII differed in 8 of the 66 isolates (the 8 isolates were in six groups).

CHEF electrophoresis of genomic DNA, combining results obtained separately with *Sma*I and with *Apa*I, identified 47 different groups. Eleven groups contained more than one isolate (ranging from two to six isolates). One group contained an isolate from two hospitals in different geographic areas. The use of *Sma*I alone identified 46 groups, and the use of *Apa*I alone identified 44 groups; results with *Sma*I and *Apa*I differed in 3 of the 66 isolates.

Grouping results of REA of plasmid DNA (combining *Eco*RI and *Hin*dIII) differed from grouping results with whole-plasmid analysis in 20 of the 66 *E. faecium* isolates. Results with CHEF electrophoresis (combining both *Sma*I and *Apa*I) differed from results with REA (combining both *Eco*RI and *Hin*dIII) in 17 of the 66 *E. faecium* isolates.

E. faecium isolates from a patient in Virginia and a patient in North Carolina were found to be identical by both CHEF electrophoresis and REA of plasmid DNA, indicating the possibility of strain dissemination between geographically distinct hospitals. Possible intrahospital strain dissemination (isolates identical by both CHEF electrophoresis and REA) was identified in four hospitals (in California, Virginia, Georgia, and North Carolina) (Table 1). Possible plasmid dissemination (isolates identical by REA of plasmid DNA but different by CHEF electrophoresis profiles) occurred twice in isolates from geographically distinct hospitals and twice in separate isolates from patients in the same hospital.

DISCUSSION

Investigation of the epidemiology of enterococcal infections has become of greater interest and importance because of the relative paucity of antibiotics active against enterococci, recent development of multiple antibiotic resistance, and need for bactericidal activity (hence, combination of antibiotics) to achieve successful outcomes for some infections, e.g., endocarditis. Older methods of typing enterococci, e.g., antibiograms and biotyping, do not adequately distinguish separate strains.

Analysis of DNA has been used in recent years to attempt

TABLE 1. Summary of DNA typing methods for *E. faecium* clinical isolates by geographic area

Geographic area (no. of isolates)[a]	No. of groups determined by:						
	CHEF electrophoresis			Plasmid analysis			CHEF electrophoresis and plasmid analysis
	*Sma*I	*Apa*I	Combined enzymes	*Eco*RI	*Hin*dIII	Combined enzymes	
California (15)	8	7	8	11	10	12	13
Rhode Island (6)	3	3	3	4	6	6	6
Virginia (13)	10	10	10	9	9	9	11
Illinois							
Hospital A (5)	5	5	5	5	5	5	5
Hospital B (4)	4	4	4	4	4	4	4
Michigan							
Hospital A (3)	3	3	3	3	3	3	3
Hospital B (6)	5	5	5	6	6	6	6
North Carolina (5)	3	3	3	4	4	4	4
Georgia (2)	1	1	1	1	1	1	1
South Carolina (1)	1	1	1	1	1	1	1
Vermont (1)	1	1	1	1	1	1	1
Toronto, Canada (5)	4	4	4	4	4	5	5

[a] Isolates from a patient in Virginia and a patient in North Carolina were identical.

to distinguish strains. A commonly used DNA typing method for enterococci has been plasmid analysis. Whole-plasmid analysis in combination with restriction endonuclease digestion of purified plasmid DNA has continued to be a very useful tool in many investigations, including those of nosocomial infections involving ampicillin-resistant *Enterococcus raffinosus* and β-lactamase-producing *E. faecalis* with high-level resistance to gentamicin (1, 3, 8, 13, 19–21, 25).

Plasmid analysis has been complicated by technical problems such as difficulty in extracting plasmids from enterococci or gain, loss, or recombination of plasmids from strains, resulting in inconsistent reproducibility. Circular forms of plasmid DNA and chromosomal-DNA contamination complicate interpretation of bands. Furthermore, some enterococcal strains lack plasmids altogether. REA of plasmids increases ability to distinguish strains but is dependent on ability to reproducibly harvest plasmids.

CHEF electrophoresis has been used increasingly in recent years for typing enterococci, especially in epidemiologic studies of *Enterococcus faecalis* and *E. faecium* (1, 3, 11, 14, 15). This method is able to separate large DNA fragments of up to 10 megabases in agarose gels by using alternately pulsed, perpendicularly oriented electrical fields from several directions. The durations of the pulses can also be varied throughout the analysis in order to better separate DNA fragments of different sizes. CHEF electrophoresis has become popular because it is not dependent on plasmid extraction, provides consistent reproducibility, and enhances resolution of DNA bands, allowing ease of and confidence in interpretation. Our experience in this study was consistent with the foregoing; interpretation of chromosomal bands by CHEF electrophoresis was done much more easily than interpretation of whole plasmids or restriction enzyme-digested plasmids. Reproducibility of results was also much better with CHEF electrophoresis than with plasmid analysis.

In the present study, CHEF electrophoresis identified 11 groups that contained more than one isolate, totaling 30 isolates. Only 1 of these 11 CHEF electrophoresis-defined groups contained isolates from different hospitals. Isolates from the same hospital are more likely to represent a single strain than are isolates from geographically distinct hospitals (14, 19, 20). The CHEF electrophoresis results are very consistent with this schema, providing evidence that CHEF electrophoresis has good discriminatory power and will be useful for looking for evidence of strain dissemination between hospitals. The addition in this study of a second restriction enzyme differentiated a few more *E. faecium* strains than did either enzyme used alone (*Sma*I or *Apa*I) (Fig. 1). Although this occurred with only a small percentage of the total isolates, the use of more than one enzyme may be useful in selected clinical situations such as nosocomial outbreaks of resistant strains, where enhanced differentiation is important.

Our study found that possible intrahospital and interhospital plasmid dissemination occurred but was uncommon, which is consistent with a previous study of gentamicin-resistant *E. faecalis* showing infrequent interhospital plasmid dissemination (19). Our findings also suggest that while strain dissemination between patients in the same hospital is not uncommon, possible strain dissemination between hospitals in geographically distinct areas is rare. This contrasts

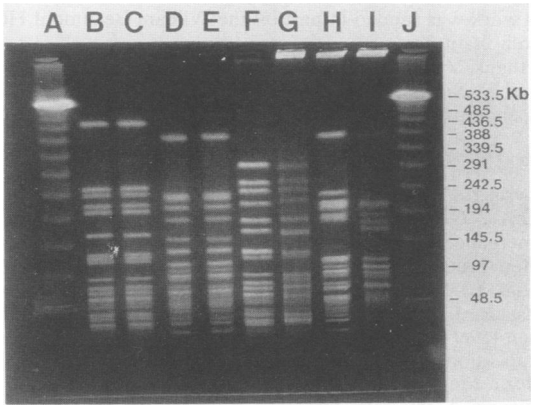

FIG. 1. CHEF electrophoresis of *E. faecium* isolates. Examples show two isolates identical by *Sma*I digestion (lanes B and C) that are also identical by *Apa*I digestion (lanes D and E) and two isolates very similar by *Sma*I (lanes F and G) but different by *Apa*I (lanes H and I). Lanes A and J, lambda ladder standards.

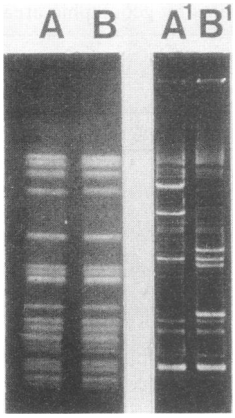

FIG. 2. Example of two isolates identical by CHEF electrophoresis (lanes A and B; *Sma*I digested) but different by plasmid analysis (lanes A^1 and B^1; *Eco*RI digested).

with evidence for clonal spread in one strain of β-lactamase-producing *E. faecalis* to six hospitals in diverse geographic areas (15). The high diversity of strains and their different plasmid patterns in the present study suggest that non-β-lactamase-mediated ampicillin resistance in *E. faecium* is not due to the spread of a single strain or a single plasmid.

The differences between whole-plasmid analysis and REA of plasmid DNA confirm that these two methods should be used together. In evaluations of plasmid epidemics, whole-plasmid analysis would be crucial. There was notably improved plasmid differentiation when two restriction enzymes (*Eco*RI and *Hin*dIII) rather than either enzyme alone were used. Comparative analysis of CHEF electrophoresis and plasmid typing results showed differences between the two methods (Fig. 2). Thus, utilizing CHEF electrophoresis alone for grouping *E. faecium* may not be sufficient, particularly when a plasmid epidemic or a resistance determinant is being evaluated. The combined use of whole-plasmid analysis, REA of plasmid DNA with two enzymes, and CHEF electrophoresis of genomic DNA with two restriction enzymes should be considered when *E. faecium* is typed for epidemiologic investigation.

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